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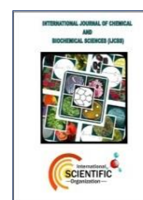

Biological Activities of Aqueous Extract of *Caralluma europaea*

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Abstract

Caralluma europaea is a medicinal plant used in Moroccan popular medicine and is recognized as a remedy with various therapeutic effects. The aim of this study was to evaluate the effects of *C.europaea* aqueous extract on neutrophil bactericide and on platelets aggregation, as well as antioxidant and hemolytic activities. The phytochemical constituents of *C. europaea* were determined by different qualitative tests. The effect on neutrophil bactericidal activity was assessed using MTT colorimetric bactericidal assay. Moreover, its inhibition of collagen-induced platelet aggregation has been investigated. The antioxidant power was determined using both DPPH and FRAP assays, and the hemolytic effect of *C.europaea* was evaluated by quantifying hemoglobin rates. The chemical analysis showed the presence of bioactive constituents such as flavonoids, saponins, coumarins and catechic tanins. IC_{50} of DPPH scavenging activities and EC_{50} of FRAP capacities was 1.44 and 4.22 mg/mL, respectively. Based on DPPH assay, the IC50 values are 1.44 vs. 0.30 mg/ml control ($p<0.05$). The EC_{50} values of FRAP assay were 4.22 *vs.* 0.083 mg/ml. The *C. europaea* extract showed significant dose-dependent antihemolytic property, and the bactericidal action of neutrophil was also suppressed. Finally, inhibition of collagen induced platelets aggregation treated with 500 µg/ml of *C.europaea* extract observed. The present results suggest that the aqueous extract of *C. europaea* possess antioxidant, anti-hemolytic and neutrophil bactericidal activities, and it is also an anti-aggregant agent.

Keywords: Antioxidant, Anti-hemolytic, Anti-aggregant, *Caralluma europaea*, Neutrophil bactericidal activity.

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1. Introduction

Traditional medicine is attracting more interest and its use has increased over the past three decades. According to the World Health Organization (WHO), more than 80% of African people still rely on popular medicine for their benefit care including cancer treatment [1]. Nowadays, many pharmaceutical agents have been sourced in medicinal plants. However, the potential of plants as sources for the production of new drugs is largely untapped [2]. Medicinal plants play an important role in folk medicine, and they have been described in worldwide pharmacopoeia [3]. *Caralluma europaea* is a wild species frequently used in Moroccan traditional medicine for its presumed anticancer activity [4]. *C. europaea* is a member of the family of Apocynaceae, and it is widely spread in Southern Jordan and the North African coast of the Mediterranean Sea [5]. In Morocco, *C. europaea* is used in traditional medicine for its anti-inflammatory, antipyretic, antinociceptive, antihyperglycemic, antidiabetic, antitrypanosomal, neuroprotective, and antiparasitic activities [6]. Several scientific studies have confirmed the antioxidant, antimicrobial, and anti-inflammatory activities of *C. europaea* [7, 8]. In traditional medicine, the properties of plant extracts depend on the part of the plant used as well as the extraction solvent. Aqueous extract of *C. europaea* have been shown to be rich in flavone glycosides such as luteolin 4′-neohesperidoside, luteolin-3′-O-(6′-O-sinapoylglucoside)-4′- O-neohesperidoside, and luteolin-3′-O-(6′-O-feruloylglucoside)- 4′-O-neohesperidoside; monoterpenoids; α-terpinene; linalool; and terpinolene [5, 9]. The objective of the present study was to evaluate the effect of aqueous extract of *C. europaea* on Neutrophil bactericidal, antioxidant and hemolytic activities. Moreover, we aimed to investigate the platelet aggregating inhibitory effects of *C. europaea* extract.

2. Materials and Methods

2.1. Preparation of Caralluma europaea aqueous extract

C. europaea (Guss) has been harvested in Beni Mellal region, Morocco (32° 19' 48" North, 6° 21' 0" West) and identified at the laboratory of Immunology and Biodiversity (LIB) of the Faculty of Sciences in Casablanca, Morocco. The aerial part of plant has been dried at the shadow for 15 days. Extraction was carried out by decoction of 50 g of *C. europaea* powder in 500 ml of distilled water and heated under reflux at 60°C for 1h. The mixture was then centrifuged, filtered and evaporated at 40°C under reduced pressure using rotary vacuum evaporator. The extract was then stored at -20°C until use. On the day of the experiments,

the crude extract was dissolved in distilled water at the described concentrations.

2.2. The phytochemical screening

The aqueous extract was subjected to qualitative chemical screening for identification of various classes of active chemical components. For the detection of phenols, 2 ml of distilled water followed by a few drops of 10% of ferric chloride, was added to 1ml of aqueous extract. The appearance of blue or green color indicated the presence of Phenols. In order to detect flavonoids, 0.5 ml of a solution of NaOH (2N) was added to 1ml of aqueous extract. The presence of flavonoids is defined by the apparition of a yellow color. To detect tannins, 5ml of distilled water and 1ml of a 5% solution of $FeCl_3$ were added to 0.5ml of aqueous extract. The appearance of a dark blue color indicated the presence of Gallic tannins. A dark green color defined the presence of catechic tannins. While to detect saponins, 1 ml of aqueous extract was added to distilled water in a test tube. The test tube was shaken horizontally for 10 seconds. The stable presence of foam defined the presence of saponins. Test for coumarins, 1ml of 10% of NaOH was added to 1ml of aqueous extract. The presence of coumarins is defined by the apparition of a yellow color. For the detection of flavones aglycones, 2 particles of mg metal and a few drops of HCl contrate were added to 1ml of heated aqueous extract. The presence of flavones aglycones is defined by the apparition of a red-orange color [10].

2.3 Determination of Antioxidant Activity
Free Radical-Scavenging Ability (DPPH)

The 2,2-diphenyl-1-1-picrylhydrazyl (DPPH) free radical scavenging method was used for the determination of the antioxidant activities of the aqueous extract of *C. europaea* as reported by Brand-Williams et al [11, 12]. To 50 µl of aqueous extract at different concentrations (0.1, 0.375, 0.75, 1.5 and 3 mg/ml) or ascorbic acid (positive control) at different concentrations (0,075; 0,09; 0,2; 0,4; 0,6; 0,8 and 1 mg/ml) were added to 1950 µl of methanol DPPH solution and incubated at room temperature, in the dark for 30 minutes. Absorbance was then measured at 517 nm. All the analysis were triplicated. The blank solution consisted of methanol DPPH solution. Antiradical DPPH activity was expressed as IC_{50} in mg/mL; which denoted the concentration of sample required to scavenge 50 % DPPH free radicals. The scavenging activity was estimated based on the percentage of DPPH radical scavenged using the following equation:

$$\% \text{ Inhibition} = \left(\frac{\text{A0} - \text{A1}}{A0}\right) x100$$

Where A0 was the absorbance of the control and A1 was the absorbance of the sample.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP method determines the capacity of antioxidants to reduce Fe^{+3} to Fe^{+2}. 1 ml of extract was dissolved with 2.5 ml of phosphate-buffered saline and 2.5 ml of potassium ferricyanide (1%). After incubation for 20 min at 50°C, 2.5 ml of trichloroacetic acid (10%) was added. The obtained solution was centrifuged for 10 min at 3000 rpm. Then, 2.5 ml of the supernatant was combined with 0.5 ml of $FeCl_3$ (0.1%) and 2.5 ml of distilled water. The absorbance was determined spectrophotometrically at 700 nm.

2.4 In vitro hemolytic activity assay

Hemolytic assay was carried out as described by Bulmus et al [13]. Whole human blood was collected in EDTA vacutainers after informed consent was obtained from all volunteers. The plasma was collected after centrifugation for 15 min at 2500 rpm and the human red blood cells (HRBCs) were washed three times with a solution of NaCl (150 mM). The HRBCs were re-suspended in 100 mM phosphate buffer solution (PBS), and diluted to 10% of their initial concentration to have a final concentration of 10^8 HRBCs per 200 µl. Then, 800 µl of PBS was mixed to 200 µl of HRBCs and to 50 µl of different concentrations of aqueous extract of *C. europaea.* After 1 h of incubation at 37°C, the tubes were centrifuged at 13500 g for 5 min. The supernatant was collected and the absorbance measured at 540 nm. HRBCs incubated with Triton-X 100 (1%) were used as the positive control, and PBS alone was used as negative control. Each experiment was performed in triplicate and inhibitory activity of the extract was calculated using the following equation and expressed as percent of hemolytic activity:

$$\% \text{ Hemolytic activity} = \left(\frac{\text{Abs sample} - \text{Abs Cn}}{\text{Abs Cp} - Abs\ Cn}\right) x100$$

2.5 Neutrophil bactericidal activity assay
Isolation of human neutrophil Human

Polymorphonuclear neutrophils cells (PMNs) used in this study were isolated from peripheral blood from healthy volunteers using the original method of Boyum [14] modified by Kobayashi et al [15]. PMNs were obtained from heparinized venous blood by Ficoll-Paque (1.077 g/liter, Pharmacia) centrifugation, followed by Dextran 2% T-500 (Pharmacia) sedimentation and hypo-osmotic lysis of erythrocytes and they were maintained in RPMI 1640 medium (GIBCO), buffered with 10 mM Hepes, and incubated on ice until used. PMNs in each preparation were enumerated visually on a hemacymeter in 2% acetic acid, and slides were routinely prepared and stained with a modified Wright-Gigemsa (Sigma). Each PMN preparation contained 95–98% of neutrophils, with the remaining cells being predominantly eosinophils.

Degranulation study

fMLP at 10^{-6} mol/L was used as a stimulant of PMN degranulation [16] and superoxide anion production [17]. Lysozyme release was measured by *Micrococcus lysodeikticus* lysis, spectrophotometrically monitored at 450 nm, and expressed as the percentage of total cellular activity [18].

2.6 Colorimetric bactericidal assay

Colorimetric bactericidal assay was performed using *Staphylococcus aureus* ATCC43300 strain (pre-cultured for 18h at 37°C in Nutrient Broth; 1 x 10^8 Bacteria /ml), it was maintained with autologous inactivated human serum in RPMI 1640 (Dubelco). Fifty µl /well of opsonized *S. aureus* were then added to the Neutrophils treated or not with *C. europaea* extract. Opsonized bacteria were co-incubated in the plates to enable the construction of a standard curve of bactericidal activity. Followed by Triton 0.2% X-100 lysis of neutrophils. The bacteria and Neutrophils were triturated

in the well. After treatment, 50 µL of MTT solution at 2 mg/mL were added to each well and incubated for 10 min at 37°C. The supernatant was removed and 150 µL DMSO were added per well. After shaking microplates After shaking microplates, 50 µl of PBS was finally added to solubilize the remaining formazan. Quantification of formazan produced by bacteria was performed by measuring optical density (OD) at 560 nm. OD corresponding to 0 - 90 % killing bacteria was established by linear regression analysis using standard curve. Positive control consisted of incubating Neutrophils with opsonized bacteria alone. Percentage of killed bacteria was determined using the following formula [10]:

$$\%\ \text{of killed bacteria} = 1 - \left(\frac{\text{Abs sample} - \text{Abs 90\% Killing}}{\text{Abs 0\% Killing} - \text{Abs 90\% Killing}}\right) x100$$

2.7 Platelet aggregation assay

Aggregation was monitored on an eight-channel optical aggregometer (SD Medical Innovation, Frouard, France) at 37°C under stirred conditions as previously described [19, 20]. Briefly, the samples were stimulated with collagen (Chrono-Log, USA) at concentration of 2 µg/mL. The dose 500 µg/mL of the aqueous extract of *C. europaea* was tested in this experiment. Platelet aggregation was then monitored following the addition of an appropriate concentration of collagen and recorded until trace stabilization and light transmission was measured at the time of maximum aggregation. As a control, a free *C. europaea* extract tube was used.

2.8 Statistical analysis

All experiments were analyzed at least three times, and results are shown as mean ± standard deviation. Analysis of the dose-response curves and the IC_{50} values was done and statistical significance of the experimental results (significance level of $P < 0.05$) was calculated using prism 8 software for Windows (GraphPad Software Inc).

3. Results and Discussion

Traditional medicine is gaining popularity as a source of complementary and alternative therapies. In contrast to conventional pharmaceuticals, it generally presumed as safe and without side effects [21]. This work aims to evaluate the effects of the aqueous extract of the medicinal species *C. europaea* on different activities directly associated with human preventive therapy.

3.1 The phytochemical screening

The results of the phytochemical analysis of aqueous extracts of *C. europaea* revealed the presence of flavonoids, catechic tannins, saponins and coumarins, (Table 1). The qualitative phytochemical study showed the presence of different classes of bioactive secondary metabolites, such as flavonoids, saponins, coumarins, and catechic tannins, formerly described to have various medicinal activities [22]. These compounds contributed to the antioxidant properties, known to be very active scavengers of many free radicals [23] and they can play an important role in the initiation of deleterious free radical actions [24]. Species of the genus *Caralluma* have been known for their richness in phenolic compounds [4, 25].

3.2 Assessment of antioxidant activity using FRAP and DPPH assays

The antioxidant activity of *C. europaea* extract was determined using two assays, namely DPPH and FRAP. The results vary according to the used methods. For DPPH assay, the percentage of free radical inhibition of aqueous extract of *C. europaea* was lower than that of ascorbic acid (Table 2). The antioxidant activity of the aqueous extract and ascorbic acid were IC_{50} =1.44 and 0.030 mg/ml, respectively (Table 2). Moreover, the reducing power assay showed lower antioxidant activities of the aqueous extract of *C. europaea* than the standard (ascorbic acid). The EC_{50} values of aqueous extract and ascorbic acid were 4.22 and 0.083 mg/ml, respectively (Table 2).

DPPH test is a method for estimating free radical scavenging activity of antioxidants, it is based on the ability of the extracts compound to give a hydrogen atom, reducing the purplish color of DPPH to yellow. The FRAP test determines the ability of these complexes to break the free radical chain by donating an atom of hydrogen [26]. In the present study, the antioxidant activity of the aqueous extract of *C. europaea* was determined using DPPH and FRAP assays. The *C. europaea* extracts showed a lower antioxidant power compared to ascorbic acid. The IC_{50} and EC_{50} values of the aqueous extract were 1.44 mg/ml and 4.22 mg/ml, respectively, which were significantly higher than that of the ascorbic acid (0.030 and 0.083 mg/ml).

3.3 In vitro hemolytic activity test

Several plants have serious adverse effects, which include hemolytic activity. This experiment aimed to assess whether *C. europaea* extract produced hemolysis of human erythrocytes *in vitro*. The results are presented in Figure 1. The extract seems to have an anti-hemolytic effect at low concentration 50 mg/ml (1.83%). Anti-hemolytic effect increases (2 and 4%) with increase of concentrations (50 and 800mg/ml), respectively. HRBCs were re-suspended in 100 mM phosphate buffer solution (PBS) at a final concentration of 5x108 RBCs/ml. Then, 800 µl of PBS was mixed to 200 µl of RBC and to 50 µl of different concentrations (50; 200; 400 and 800 mg/ml) *C. europaea* extract. After 1h at 37°C of incubation the tubes were centrifuged. The supernatant was collected, and the absorbance measured at 540 nm. HRBC incubated with Triton-X100 (1wt %) were used as the positive control, and PBS alone.

Table 1: Results of phytochemical screening of aqueous extract of *C. europaea*.

Compounds	Aqueous extract of *C. europaea*
Flavonoids	+
Saponins	++
Coumarins	+
Catechic tannins	++

++: abundantly present. +: present.

Table 2: Antioxidant activity of *C. europaea,* expressed in mg/mL.

	C. europaea	Ascorbic acid
DPPH IC_{50} (mg/mL)	1.44 ± 0.19^a	0.030 ± 0.009^b
FRAP EC_{50} (mg/mL)	4.22 ± 0.56^a	0.083 ± 0.005^b

Values are expressed as mean ± SD of triplicate measurements. Means with different letters in the same column represent significant differences at $p < 0.05$.

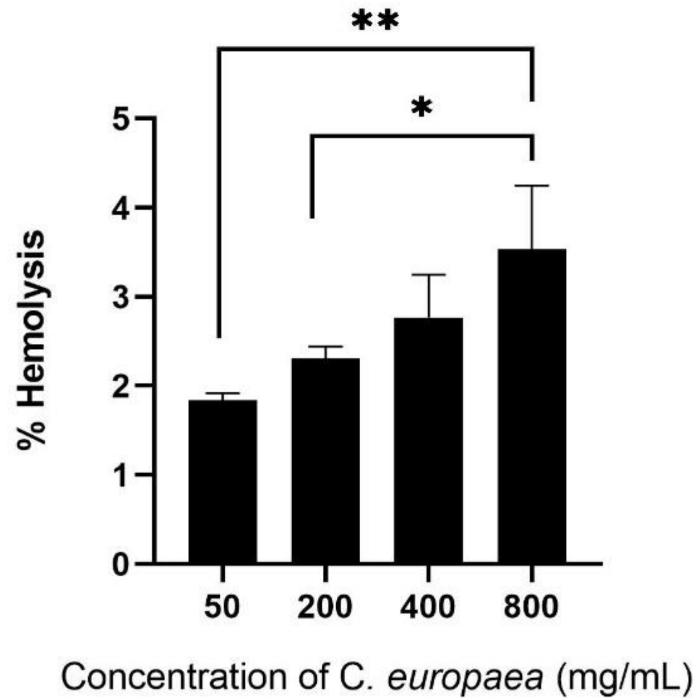

Figure 1: Hemolytic activity of aqueous extract of *C. europaea* on Human Red Blood Cells (HRBC) in vitro. Each value is represented as mean ± SD (n=3).

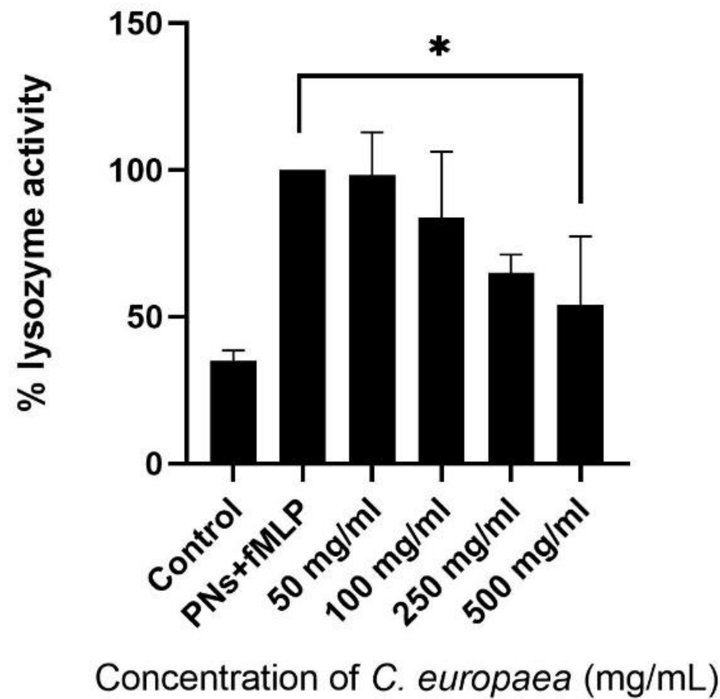

Figure 2: PMN function. Shown is the effect of the aqueous extract of *C. europaea* on lysozyme activity by PMNs stimulated with fMLP (10^{-6} mol/L). Total lysozyme activity in each experimental condition is expressed as ± SD of 3 experiments. *$p < 0.05$, as compared with FMLP (100%).

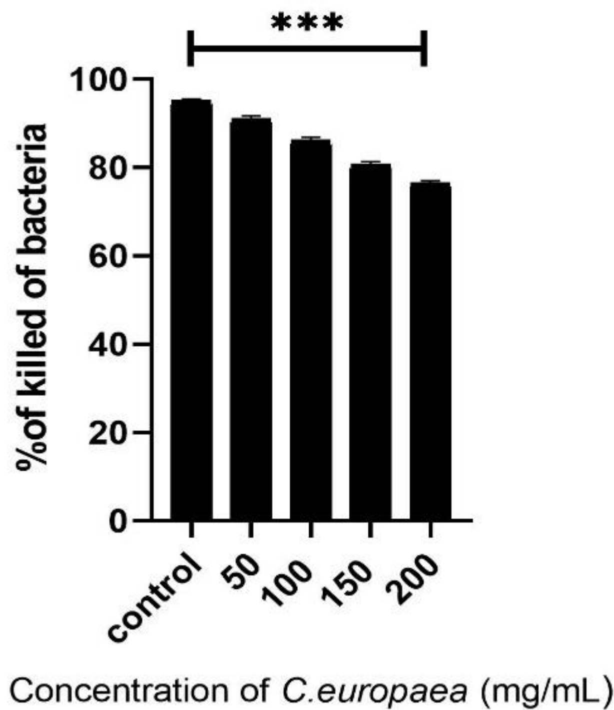

Figure 3: *In vitro* effect of aqueous extract of *C. europaea* on human Neutrophil bactericidal activity. Results were expressed as mean SD (n=3). *$p < 0.05$, as compared with control.

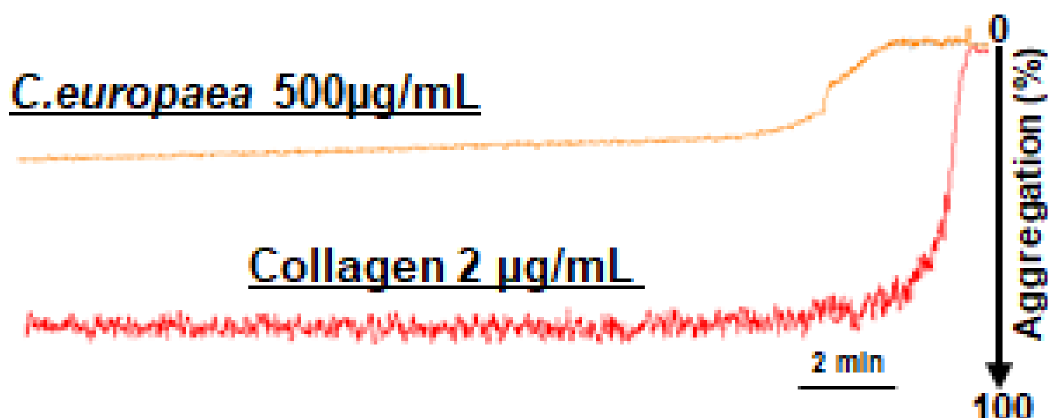

Figure 4: Original tracing showing the effect of *C. europaea* on platelets aggregation inhibition induced by collagen. Platelets were incubated with different dose of aqueous extract of *C. europaea* 500 µg/ml for 5 min at 37°C and aggregation was induced by collagen (2µg/ml). The control response was platelets aggregation induced by collagen without *C. europaea* extract. Aggregation curves are representative of 3 independent experiments.

Moreover, it is well known that the phenols and flavonoids present in the extract may inactivate free radicals, thus decreasing oxidative hemolytic activity. The mechanisms that could lead to erythrocytes hemolysis is lipids and proteins oxidation, and specifically, lipids peroxidation. Lipid peroxidation is a free radical chain reaction, leading to a damage of the erythrocyte's membrane and thus, hemolysis [27]. Our study also demonstrated that the aqueous extract of *C. europaea* exerted an anti-hemolytic activity, anti-hemolytic effect increases with increases of concentrations.

3.4 Degranulation study

PMN Degranulation Lysozyme activity by the aqueous extract of *C. europaea* with (50-500 mg/mL) in response to fMLP was 100% of total cellular activity. When PMNs were preconditioned with the extract and then stimulated with fMLP at 10^{-6} mol/L, lysozyme activity significantly decreased in a dose-dependent manner (Figure 2). PMN preconditioning with *C. europaea* did modify fMLP-stimulated PMN degranulation, which was 54% of the value for 500 mg/mL of the extract.
Furthermore, it has been shown that PMN–vascular wall interactions may play a pathological role in different situations. In these different situations, the interaction involves mainly PMN adhesion to the stimulated endothelium, followed by the activation of PMNs, including an oxidative burst [28] and the release of granule contents [29]. We used fMLP, a classic extracellular compound able to activate PMNs, to induce the oxidative burst and the release of granule contents [30]. The tested extract of *C. europaea* can limit PMN activation.

3.5 Neutrophil bactericidal activity assay

Incubation of Neutrophils for 30 minutes with 0.05; 0.1; 0,15 and 0.2 g/ml of aqueous extract of *C. europaea* exhibited an inhibition of Neutrophil bactericidal activity. The results are presented in Figure 3. This inhibition occurs in a dose-dependent manner as it increases with increase of *C. europaea* concentration of the extract. In addition, Neutrophil treated with 0.15 g/ml and 0.1 g/mL were able to kill 80.67 ± 0.63% and 86.06 ± 0.75 of bacteria, and those treated with 0.2 g/ml showed a killing rate of 76.49 ± 0.52%. Treatment with 0.05 g/ml (91.00 ± 0.68 % of killed bacteria) showed no significant effect compared to untreated Neutrophil bactericidal activity (95.09 ± 0.43 % of killed bacteria). Significant suppression ($p<0.05$) of bactericidal activity compared to untreated Neutrophils. Neutrophils were incubated with the aqueous extract of *C. europaea* for 30 min before adding *S. aureus*. Neutrophils were lysed using Triton X 100 and MTT was added to evaluate the number of surviving bacteria.

Furthermore, PMNs were the first line of defense in response to invading microorganisms, with the presence of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, high numbers of bactericidal reactive chemical species: the oxidative burst [31, 32]. Decrease of Neutrophils bactericidal activity showed in this study can be explained by the presence of antioxidants in the aqueous extract of *C. europaea*. In according to Ciz et al [33] , these results showed similar results by demonstrating that flavonoids inhibit respiratory burst of Neutrophils.

3.6 Effect of aqueous extract of C. europaea on human platelets aggregation

In order to check the physiological status of platelets, a control aggregation (free from plant extract) induced by collagen was systematically achieved at the beginning of each experiment. Washed platelets were separately pre-incubated with *C. europaea* extract for 5 min at 37°C and then the aggregation was induced. Figure 4 displays an original tracing of collagen induced platelets aggregation with and without *C. europaea* extract.

Platelet aggregation is a complex process influenced by many elements. Collagen (a strong thrombogenic component) stimulates platelet aggregation and induces the activations of various intracellular mediators (ADP, thromboxane A2, Calcium) through receptors glycoproteins (GP IaIIa, GP VI) [34]. Furthermore, platelet aggregation can be blocked by inhibiting the thrombin, ADP, phosphodiesterase and thromboxane pathways activation platelets [35]. In this study *C. europaea* extract inhibits collagen induced platelets aggregation with the concentration 500µg/mL. These results are consistent with other studies that have shown that flavonoids inhibit platelets adherence, aggregation and secretion [36].

4. Conclusion

In conclusion, the present investigation has shown that the aqueous extract of *C. europaea* possess antioxidant, anti-hemolytic and neutrophil bactericidal activities, and it has also an anti-aggregant effect in which flavonoids could be involved. Further clinical studies are needed to confirm the therapeutic potential of the aqueous extract of *C. europaea*.

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